Aliskiren inhibits renin-mediated complement activation

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Certain kidney diseases are associated with complement activation although a renal triggering factor has not been identified. Here we demonstrated that renin, a kidney-specific enzyme, cleaves C3 into C3b and C3a, in a manner identical to the C3 convertase. Cleavage was specifically blocked by the renin inhibitor aliskiren. Renin-mediated C3 cleavage and its inhibition by aliskiren also occurred in serum. Generation of C3 cleavage products was demonstrated by immunoblotting, detecting the cleavage product C3b, by N-terminal sequencing of the cleavage product, and by ELISA for C3a release. Functional assays showed mast cell chemotaxis towards the cleavage product C3a and release of factor Ba when the cleavage product C3b was combined with factor B and factor D. The renin-mediated C3 cleavage product bound to factor B. In the presence of aliskiren this did not occur, and less C3 deposited on renin-producing cells. The effect of aliskiren was studied in three patients with dense deposit disease and this demonstrated decreased systemic and renal complement activation (increased C3, decreased C3a and C5a, decreased renal C3 and C5b-9 deposition and/or decreased glomerular basement membrane thickness) over a follow-up period of four to seven years. Thus, renin can trigger complement activation, an effect inhibited by aliskiren. Since renin concentrations are higher in renal tissue than systemically, this may explain the renal propensity of complement-mediated disease in the presence of complement mutations or auto-antibodies.

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Renal diseases may be associated with activation of the alternative pathway of complement due to mutations in genes encoding complement regulators or proteins and circulating autoantibodies,[1,2] allowing uncontrolled complement activation and resulting in deposition of components of the alternative pathway and terminal–complement complex in the kidneys.[3,4] These conditions include atypical hemolytic uremic syndrome[1] and C3 glomerulopathy, the latter further subdivided into dense deposit disease (DDD) and C3 glomerulonephritis,[2] conditions that may be associated with circulating C3 nephritic factor (C3NeF), an autoantibody directed to the C3 convertase.[5,6] All these conditions ultimately lead to renal failure.

Complement-mediated disease has a propensity to affect the kidneys. In atypical hemolytic uremic syndrome disease activity subsides as renal failure progresses but recurs upon renal transplantation, indicating that viable renal tissue may contribute to disease activity. It is as yet unclear why the kidney is a target organ in these conditions. In this study we investigated whether renin, known to be secreted solely in the kidney,[7] activates the alternative pathway of complement.

Renin is an aspartate-protease released exclusively by cells of the renal juxtaglomerular apparatus.[8] Renin initiates the renin–angiotensin–aldosterone system (RAAS) by cleaving angiotensinogen into angiotensin I. To date, the only known substrate of renin is angiotensinogen.

Aliskiren is an orally active, nonpeptide renin inhibitor approved for treatment of hypertension. It has also been successfully used to reduce proteinuria in diabetic[9] and nondiabetic nephropathy[10] both in adults and children, as well as for the control of pediatric hypertension.[11] It may thus have a role in nephroprotection during chronic progressive nephropathies.

In this study we identified C3 as a novel substrate of renin and showed that renin-mediated C3 cleavage was identical to that induced by the C3 convertase *in vitro*. Renin-mediated C3 cleavage was specifically blocked by the renin inhibitor aliskiren. The effect of aliskiren on complement activation was assessed in 3 patients with DDD demonstrating evidence for decreased systemic and renal complement activation.

RESULTS

Renin cleaves C3 into C3b and C3a *in vitro*

C3 (100 μg/ml) combined with plasma renin (4.5 pg/ml, unless otherwise stated) resulted in C3 cleavage and the appearance of a band corresponding to C3b. The cleavage of

C3 by renin was confirmed using kidney renin and recombinant renin (Figure 1a). C3 cleavage by plasma renin commenced within 5 minutes and was complete between 2 and 5 hours (Figure 1b). C3 alone did not undergo spontaneous cleavage within 24 hours (Figure 1b, lane 11). C3 incubated with renin induced the release of C3a (Figure 1c and d). C3a levels induced by renin or C3 convertase-mediated C3 cleavage were comparable (Figure 1c), as were kinetics (Supplementary Table S1). The Km was 2.97 μM for plasma renin and 2.16 μM for C3 convertase (the previously published Km of the C3 convertase was 5.86 μM).[12]

N-terminal sequencing of the cleaved product confirmed that renin cleavage of C3 occurred at the same site as cleavage by C3 convertase,[13] because the N-terminal 9 amino acids of C3b α chain were identical (Figure 1e). The amino acid sequence at the cleavage site differed from the sequence within angiotensinogen.[14]

Experiments similar to those carried out with human proteins were carried out using murine renin and murine C3, but C3 cleavage could not be demonstrated (Supplementary Figure S1).

Inhibition of renin-mediated C3 cleavage by aliskiren, pepstatin, and zinc

C3 cleavage by plasma renin (0.45 pg/ml) was inhibited by the renin inhibitor aliskiren at concentrations of ≥0.01 M (Figure 1f). Pepstatin A, an inhibitor of aspartate proteases, also inhibited plasma renin (Figure 1f) and kidney renin–induced C3 cleavage at concentrations of ≥0.3 mM. Aliskiren and pepstatin alone had no effect on C3 (Figure 1f).

Cathepsin D is also inhibited by aliskiren[15] and pepstatin[16] but did not cleave C3 at a concentration of 10 μg/ml (data not shown). $ZnCl_2$, known to inhibit aspartate proteases,[17] inhibited plasma renin–induced C3 cleavage, but $MgCl_2$ and $NiCl_2$ did not (Supplementary Figure S2).

Aliskiren inhibits C3 cleavage by renin in the presence of serum

The cleavage reaction induced by plasma renin (2.7 pg/ml) was carried out in the presence of normal human sera ($n = 6$). A band corresponding to C3b was not demonstrated (Figure 2, lane 2). The lack of a band corresponding to C3b could be due to the presence of complement factor I (CFI) allowing C3b degradation. The incubation was therefore performed in CFI-depleted serum ($n = 3$). Under these conditions a band corresponding to C3b was visualized (Figure 2, lanes 4 and 7 in the presence of plasma renin and lane 8 in the presence of recombinant renin). The band corresponding to C3b was not visible when CFI was added to the CFI-depleted serum (Figure 2, lane 5). Aliskiren, at concentrations at or above 0.13 M, inhibited C3 cleavage induced by plasma renin visualized in CFI-depleted serum (Figure 2, lane 6).

Renin-mediated cleavage of a C3 peptide

Recombinant renin (0.27 μg/ml) was incubated with a fluorescence resonance energy transfer C3 peptide, consisting of 14 amino acids covering the cleavage site. Renin-mediated cleavage was compared with the C3 convertase at various time points, showing enhanced fluorescence over time (Figure 3).

Functional assays of the C3 cleavage product

C3a as a chemoattractant. C3a is a known mast cell chemoattractant.[18] The product of C3 incubation with plasma renin significantly increased mast cell migration, in comparison with C3 alone, and this effect was inhibited by pepstatin (Figure 4). The use of aliskiren was precluded owing to excessive dilution requirements.

Analysis of the interaction between C3 and renin by surface plasmon resonance. Experiments were designed to determine whether the cleavage products C3b and C3a were formed after C3 cleavage by plasma renin. Using an anti-C3b antibody that specifically recognizes the C3b neoepitope formed after C3 cleavage, binding was detected to C3 incubated with plasma renin but not to C3 alone (Figure 5a). Anti-C3a antibody bound to C3 but not to the C3–renin cleavage product (Figure 5b), indicating that C3a was released and washed away in the presence of renin.

Factor B (CFB) binding to cleaved C3 (incubated with plasma renin overnight to ensure total cleavage) was demonstrated by adding CFB to C3, C3b, and C3 incubated with renin (Figure 5c). The results show considerably more binding of CFB to C3b and C3 cleaved by renin than to uncleaved C3. Similar results were obtained when the C3 and C3b curves were aligned before injection of CFB (Supplementary Figure S3). In the presence of aliskiren CFB did not bind to C3 (Figure 5d). Factor D (CFD) cleaved CFB bound to the C3bB complex formed after adding CFB to the cleavage product of renin (Figure 5e).

Release of factor Ba. C3 or C3 cleaved by plasma renin overnight were incubated with CFB and CFD. The results indicate that C3b, generated by renin cleavage of C3, bound to CFB, and factor Ba was released in the presence of CFD. Levels corresponded to factor Ba released in the presence of the $C3(H_2O)$ convertase formed by binding of C3 to CFB (middle column in Figure 5f). Of note, C3 digested by renin (right column, Figure 5f) contains C3 totally cleaved to C3b but no C3.

Aliskiren decreases C3 deposition on renin-producing cells. Renin-producing Calu6 cells incubated with C3 for 4 hours exhibited C3 deposition, demonstrated in Figure 6a. Preincubation of the cells with aliskiren for 18 hours before addition of C3 reduced C3 deposition (Figure 6b), which was comparable to Calu6 cells, incubated with C3, in which renin was knocked out (Figure 6c).

C4 and C5 are not cleaved by renin

Incubation of human C4 with plasma or recombinant renin for 24 hours did not lead to cleavage (silver staining, data not shown). Incubation of human C5 with plasma renin for 2 and 24 hours did not lead to cleavage (see immunoblot, Supplementary Figure S4). Likewise, a band corresponding to C5a was not detected. C5a levels were also not increased

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